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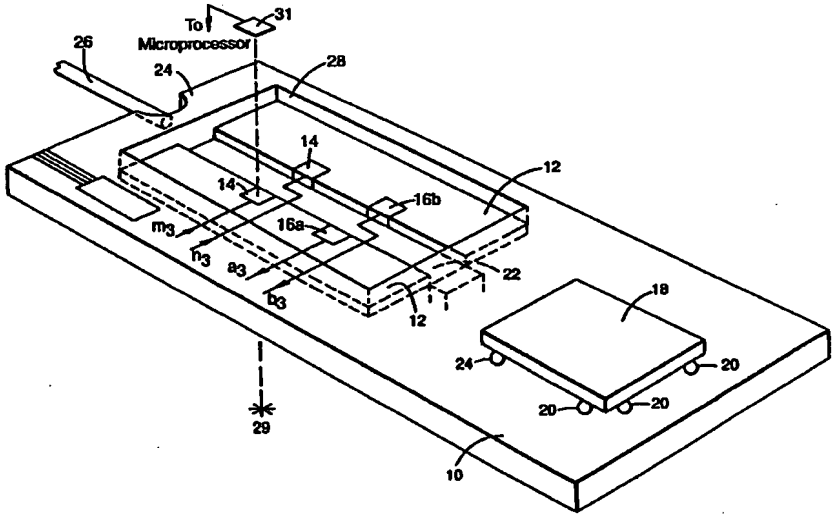

(57) Abstract

The invention relates to an apparatus for and method of introducing a substance into an object, particularly into a cell or cellular material. In a preferred arrangement apparatus receives the cell and preferably locates it between first and second electrodes and applies a voltage pulse to cause a disruption in the cell wall. This causes the cell to become permeable. The substance may then be introduced, for example under a fluid pressure. Cells may then be inspected and sorted into transfected and non–transfected types. This may be achieved automatically, for example by using electrophoresis.

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APPARATUS FOR, AND METHOD OF, INTRODUCING A SUBSTANCE INTO AN OBJECT

This invention relates to an apparatus for, and method of, introducing a substance into an object.

More particularly, but not exclusively, the invention is capable of introducing substances into small objects, such as for example, cellular material or cells. The substance introduced may be a transfecting agent such as for example: a chemical, molecule, protein, virus, prion or DNA material.

The invention will hereinafter be described with reference to cells for clarity and conciseness. However, it will be appreciated that the invention is not limited to this application and that within the specification the terms cells and objects are interchangeable.

Previously material has been introduced into cells by way of a syringe-like device. These syringe-like devices have to be very small, typically less than a few micrometres in diameter, so that they can penetrate the cell wall. Partly due to this reason, it has been very difficult to manufacture sufficiently robust syringe-like devices.

The present invention arose to overcome this and other problems associated with syringe-like devices.

According to the present invention there is provided an apparatus for introducing a substance into an object comprising: means for introducing the substance to the object; and means for causing a discontinuity in the wall of the object so as to permit said substance to enter said object; characterised in that the means for causing a discontinuity includes at least one electrode, dimensioned and arranged to form the discontinuity in the wall of the object upon application of a voltage pulse.

Preferably a characteristic dimension of a channel through, or along, which an object passes or flows is of the order of 50 μm, more preferably it is less than 25 μm.

Objects or cells are introduced into a chamber in which the apparatus(es) is/are located by way of a pump or gravity feed or other suitable fluid displacement mechanism, for example by electro-osmosis.

Preferably electrodes are provided so that the object is located with respect thereto so that a potential voltage difference may be applied in order to render the object wall temporarily permeable. Alternatively, if the object is suspended in a medium, which is at a particular voltage, only one electrode may be required; the potential difference being established between the object and the medium: This may be achieved by ensuring that a portion of the object contacts a second electrode.

Means can be provided to restrain or locate the object so that it is positioned with respect to the, or each, electrode. An advantage of locating the object is that it is positioned in a particular part of a predetermined electric field with know

characteristics. Consequently the potential difference may be applied with greater precision.

A proximity detector is advantageously included, so that when an object is in the predetermined part of the electric field, the voltage pulse is applied automatically. Processing means, including electronic logic, may be used to improve and enhance this process.

Preferably there is provided a plurality of the aforementioned apparatuses arranged in an array. An advantage of such an array is that many objects may be acted upon in parallel. This increases throughput. For the sake of simplicity, the process of acting on objects so as to introduce a substance is hereinafter referred to as transfection. Transfection includes the process of introducing a substance into an object.

An array of apparatuses may be formed on a semiconductor substrate, such as for example, silicon or germanium. Proximity detectors, electrodes and processing means may be included on the substrate, for example, in a different layer of an integrated semiconductor structure.

In a particularly preferred embodiment DNA is introduced into living cells by rupturing or forming a discontinuity in the cell wall by the process of electroporation. Cells are supported in a fluid and are able to move with respect to the electrodes.

Means for locating each object or cell with respect to an electrode may comprise a mechanical or electrical structure. An example may be a well or well-like structure, formed for example by back etching a silicon substrate in which the cell locates. A mesh or sieve-like arrangement can be placed at the exit of the well so as to permit passage of fluid but prevent the cell from leaving the well. Preferably a pressure differential established across the substrate urges cells into the well-like structures.

As more cells are located in wells the pressure differential increases because less wells become available, through which fluid may flow. This increased pressure tends to force cells into the wells as they deform relatively easily. One way of preventing this from occurring is to obtain an indication of wells which are occupied and use this information to reduce or increase the pressure differential.

This information is readily obtainable as the presence of a cell is known from proximity detectors. A counter in a microprocessor may be used to increment each time a well becomes occupied. Other detectors may be used in order to ascertain whether a well is occupied. An example is described below.

Preferably the apparatus is microfabricated from a biocompatible material. The microfabricated apparatus may include one or more microfabricated channels. These may be formed, for example, by etching in silicon dioxide. Wells or transfection sites may be at a locus of two or more fluid pathways in a fluid flow channel.

The channel is preferably narrow, for example, between 1 and 5 times the diameter of the cells (which may typically be around 5 – 10 μm) to be electroporated. Such narrow channels are advantageous in electroporation, as the electroporation voltage may be applied across the cell per se, rather than across the cell and the supporting fluid. This enables the electric field experienced by the cell to be controlled precisely. In an alternative embodiment the channel may even be narrower than the diameter of the cell in its relaxed state. In this embodiment cells deform and flow along the channel and are in closer contact with the walls.

Alternatively the channel or well may be relatively wide except for a constriction at a region at which transfection occurs, the constriction, and/or electroporation electrodes may be designed so that pores, opened in the cell membrane, to allow transfection, are preferentially oriented at a source of a transfecting agent.

In a microfabricated device electronic logic may be used to control the amplitude of the electroporation voltage pulse or sequence of pulses. The logic circuitry may be integrated within a semiconducting substrate, for example using CMOS, DMOS or bi-polar components, fabricated in a convenient process sequence. Preferably the substrate also forms a support for microfabricated channels. Post-processing techniques can be used during manufacture of the substrate to interconnect electronic components to electrode flow channel(s).

Integration of a moderately high-voltage (typically 5-25 volts) switch device (used to control the electroporation voltage) is especially advantageous as the minimal electrical

impedance between the switch and the channel may be used to enhance control of the electroporation parameters. The inclusion of one or more capacitive elements adjacent the switch is most preferable as this enhances the ability to source current rapidly, without requiring complex current distribution circuitry to the switch. This may be of particular advantage when there is integration of multiple electroporation devices for example in an array.

The introduction of electronic components or logic circuitry, for example by doping to produce an active substrate, is elegant and is of especial benefit when an array, or arrays, of electroporation devices are co-fabricated on a common substrate. The possibility exists to substitute or augment such components or circuitry by attaching additional microelectronic components, at appropriate positions, to the substrate. Such components may be attached by surface mount, die and wire bonding, TAB bonding or flip chip bonding. The attachment of devices using conductive adhesive means is especially preferred since this minimises any thermal stresses imparted to the structure during fabrication.

Preferred devices and attachment means and capacitor devices are attached by surface mount (including attachment by conductive adhesives) or by wire bonding. The aforementioned devices are particularly preferred where the substrate is passive or contains low voltage components. Analogue processing circuitry, analogue-to-digital converters, (ADC's) digital signal processing devices, microcomputing or microcontroller elements, and communications devices may also be integrated onto the substrate. The latter devices include optical communication devices. Integration

facilitates connection of processing or control circuitry to external processors, such as a microprocessor for closed loop flow control and/or electroporation pulse application. Closed loop flow control is used to increase/decrease the rate of cell occupancy of wells.

Preferably sensing means is provided which operates in conjunction with an electrode and a common ground, or a pair of electrodes, to interrogate a channel or well for determining the presence of a cell.

Preferably control means controls the instant of electroporation pulse timing, in collaboration with the detection of a cell in the well or channel. For example, a microcontroller, timer or state-machine may be integrated and used to control the instant of application and/or amplitude of an electroporation pulse, in response to a signal indicating the presence of a cell.

Preferably means is provided to determine the state or condition of cells following transfection, indicating whether a cell is unaffected, transfected or damaged. For example apparatus as described in published International Patent Application No WO-A-9402846 (BTG) may be utilised for this purpose. Thus it is possible to characterise the cell, at a locus or loci both prior to and following an attempt at transfection, and to determine the success or otherwise by the difference in the cell's response rather than by an absolute calibration.

Preferably processing means responds to an external indication of the presence or state of a cell in the electrode vicinity. A trigger may be provided by an operator or automatically by a digital input/output card in a common microcomputer. The trigger may alternatively be derived by image processing means such as a video microscope image of the channel.

Detection or monitoring of cells may be achieved by optical means. Transfected cells may have a fluorescent component introduced in them when transfection occurs. This allows automatic fluorescence detection of transfected cells, for example by using a video microscope and/or other image processing means, to detect the transfected cell. This data may be used to signal the presence of the cell in the electroporation apparatus.

Integrated components or circuitry and an associated well or channel, in the or each electroporation apparatus, are provided with a unique address and a communication means is provided allowing communication to and from a microprocessor. Preferably communication is via a common link or bus.

In the case where the support substrate is silicon the possibility exists to view the cell handling structures through the silicon using suitable infra red radiation. In such an embodiment care must be taken in the layout of the structures to prevent obscuration of the radiation path. An advantage of this embodiment is that the device need not use any member which is optically transmissive in the normal visible band, but is infra red transparent. As infra red radiation may inhibit any fluorescence detection, it is preferred to carry out any infra red detection at a separate location to fluorescence detection.

Subsequent to transfection of cells, there is preferably provided sorting means to direct cells into a collection flow or to a waste flow depending on the result of transfection. Such sorting may be achieved by a number of methods and include: electrophoresis, dielectrophoresis, "optical tweezers", and/or directed pressure pulses.

Means may also be provided to destroy cells which have not been transfected in the desired manner. Such destruction may be achieved by killing the cell(s) while leaving it/them essentially physically intact or by physically disrupting the cell(s). Preferred means of achieving this include: electrical disruption of the cell, essentially by overly vigorous electroporation, the introduction of a cell lysing agent; or rapid local heating of the cell or fluid medium in the vicinity of the cell. A micro-heating element or directed, pulsed infra red radiator or laser may be used for this purpose.

In cases where the electroporation apparatus has electrical connections, routed about or around it, for example in a highly integrated active substrate, it may be desirable to provide electrical guard bands suitably disposed around portions of a fluid handling structure so that any electric field applied to the fluid is reduced sufficiently so that there is minimal deleterious effect on cells flowing in the channel.

Optical components, such as waveguide optics, may be integrated in processed layers of the substrate which are preferably fabricated in similar processes to those defining fluid channels. Such optical components may include waveguides for interrogation of the cell or support medium in the fluid channel. These may include evanescent field coupling.

Alternatively optical components may be arranged to communicate to external signal processing means. Optical components include structures interfacing with fibre optic elements such as etched silicon having structures formed thereon, including V-grooves.

Arrays of apparatuses may utilise common external connections for supply of fluids, cells and power supplies and may be imaged in parallel using suitable video microscopy means.

Embodiments of the invention will now be described, by way of examples only, and with reference to the following Figures in which:

Figure 1 shows an overall diagrammatical view of one embodiment of an apparatus;

Figure 2 is an overall general view of a substrate incorporating an array of alternative apparatuses to those shown in Figure 1;

Figure 3 is a sectional view through the apparatus of Figure 2;

Figures 4 show sectional views through alternate fluid flow and electrode configurations;

Figure 5 is an overall view of a system incorporating the invention; and

Figure 6 is a section through an alternative electrode arrangement showing proximity detectors.

Referring to the Figures, there is shown in Figure 1 an apparatus according to the invention including a substrate 10 which is formed from semiconductor material such as silicon. Substrate 10 optionally includes a CMOS or DMOS or bipolar active devices

which is interconnected, for example as shown in Figure 5. Channels 22 are fabricated in the device in order to direct the flow of fluid and cells. An electrode pair 16a and 16b is connected to a voltage supply (not shown) by way of a switch which is activated by the active device.

The electrode pairs 16a and 16b are preferably formed from gold plated pillars. It is the electrode pair which, when energised with a voltage pulse, form a discontinuity or weakness in the cell wall so as to permit the infusion of a substance. Optionally electrodes 16 perform the function of detection of the presence of a cell, by for example variations in capacitance between the electrodes 16a and 16b. It will be appreciated that other means for the detection of cells may be used, for example these may include an optical detector.

Fluid flowing through channel 22 permits the passage of cellular matter supported in the fluid through the electrode configuration. The fluid may pass along the surface of substrate 10 into a plurality of other channels (not shown) whence it is directed to a larger channel or capilliary tube for subsequent processing. Alternatively, as shown in Figure 3, channels may be formed through the substrate. In the embodiment shown in Figures 2 and 3, cells are located in well-like structure 15 and a potential difference is applied to each cell in parallel. The advantage of the embodiment shown in Figures 2 and 3 is that a very large number of cells may be transfected at substantially the same instant.

Microelectronic logic device 18 may be formed integrally with the substrate material 10 or bonded thereto. Interconnections from device 18 may be by way of conductive glue or solder for example as is achieved in flip chip bonding. A cover plate 28 closes channel 22 substantially transparent to the wavelength of radiation from source 29. The radiation may be an infra red laser or other suitable detectable radiation and this may be used in order to detect the presence of a cell prior to transfection. Alternatively, the source of radiation may be used to detect whether a cell has been transfected and this process is described below.

An optical fibre 26 may be provided for the inspection of the channel and/or the inspection or detection of a cell.

Referring to Figures 2 and 3, when arranged in an array, it is advantageous to identify each well-like structure and its contents as well as the status of the processor 18. One way of achieving this in very large arrays (typically of the order 10s or 100s of rows and columns) is by way of ascribing individual unique addresses to each well and associated electrodes and control circuitry.

Figure 4a shows a microfabricated device 2, for example made from etched glass, silicon or moulded plastic as known in the art, which has a flow channel 40. The channel is typically between 1 and 5 times the diameter of the cells which flow through it for transfection. In this embodiment the cells are supported in a medium. Inlet and outlet connection means (recesses, plugs, sockets etc.) 60 are provided which allow capillaries or other hollow connections 80 to be connected to the flow channel. One or

more pairs of electrodes 100, 120 are provided in contact with the channel, with electrical connections to external devices via tracks 140 and contacts 160. Electroporation potentials are provided by power supplies not shown. Alternatively, an electrode pair may be configured initially in a detection mode and when a cell is detected, switched into electroporation mode. A series of electrode pairs may be provided to give sequential potential treatments. The electrodes may also be used to measure properties of the cells.

As shown in Figure 4b the electrodes may use a common ground electrode 200 instead of discrete pairs. An electroporation electrode 240 shaped in order to concentrate the field towards the cell or a particular area of the cell. Detection electrodes 220 may be shaped for maximum sensitivity or simply planar. As in Figure 4c the channel 40 may be close to or even smaller than the diameter of the cells in all or part in order to give close contact between the electrodes and the cell wall as shown in Figure 4c.

In all the above embodiments the pairs of electrodes may be on the same side of the channel or opposite sides.

Figure 4d shows an embodiment in which cells 180 are flowed into flow channel 40 from a supply reservoir not shown, in a carrier medium without the transfection material; the device 2 has a junction 330 shown as a T with a second channel 332 for supply of the material 334 to be transfected. Electrodes 338a, b and optionally 340 and 342 are electroporation electrodes. Again these may be discrete pairs or electrodes with a common ground. Again, the electroporation power supply may be a single potential

applied to each electrode pair for a given time, or may be a sequence of potentials applied to one or more pairs of electrodes. The transfection material 334 may be flowed continuously into channel 40, or may be applied as a slug only when a cell is present, so enveloping the slug during electroporation. Detection electrodes 336 upstream of the junction may control injection of the material 334 in order to achieve this; electrodes 360 may also control the electroporation process. As in Figure 4b, electrodes may be shaped in order to enhance the poration process. The position of the cell may be monitored optically using detectors (shown diagrammatically as 343) at various stages of the process. Monitoring after the process, for example to check that a fluorescent dye has been introduced into the cell along with the desired material, allows the cells to be sorted according to success or failure of transfection.

Additional side flow channels may be provided to supply to the vicinity of the cell additional materials which assist delivery of the transfection material into the cell. For example, localised chemical poration may be achieved by introduction of an appropriate chemical. This introduction may be transient and under closer control in microfluidic channels so giving advantage over the prior art. Control of the additional introduction may be achieved by electrodes or optical means.

Figure 4e shows an alternative arrangement to that shown in Figure 1 in which pressure applied to cells to be transfected assists the process. The cell 180 enters the device along a flow channel 4 entrained in a carrier medium. The cell is captured from this flow by a secondary flow along channels 52 and is localised in well 50. The cell is localised against the orifice of channel 54 which contains the transfection material 34,

by a force created by the pressure drop of the flow between channels 4 and 52, or if the cell is a tight fit to the well 50, by a static pressure difference between these channels. Alternatively, the cell may be held in place by physical force from a plunger or deformable section in the top wall 55 of channel 4. A pressure pulse applied to material 34 in channel 54 will then force 34 into the cell. The pulse may be applied for example by deformation of a membrane 60 on a chamber 56. The large ratio of area of 60 and the orifice of channel 54 will easily produce a large sudden pressure for injection.

Additional and/or optional electrodes 62, 64 may be provided to electroporate the cell membrane around the orifice of channel 54, reducing the need for a pressure pulse. Electrodes 62, 64 may also or alternatively be used to detect the presence of the cell in order to initiate or control the process. Electrodes may additionally or alternatively be placed in other positions such as on the sidewalls or around the top of the well 50, e.g. as shown in positions 66, 68, or in contact with the material 34 in order to achieve the above.

Figure 6 shows a further embodiment in which a sheath flow arrangement is used to prevent cells adhering to sidewalls and to control their behaviour using fluid flow. Cells are introduced to the device through a flow channel 100 which is joined by one or more further channels 102 to provide a sheathing laminar flow, the boudaries between the flows being shown diagrammatically at 104. Electrodes 106 with connections 108 then achieve electroporation. These electrodes may advantageously be shaped in order to concentrate field and / or localise poration. Further pairs of electrodes or other variants as described above may be incorporated. Material to be transfected 34 may be

introduced into the cell flow down channel 100 as described above, or alternatively may be introduced into the sheath flow using further channels 110. Material 34 may be introduced as slugs 106 surrounding the cells; control of introduction of 34 may be by detection electrodes 112 as before. Transfection may be monitored using electrodes or optically (122) as above; more than one outlet 116, 118 may be provided in order for cells to be sorted according to the monitor output. Such sorting processes are well known in the art.

It is understood that while a single transfection site device has been described in the above, the invention is intended to cover either single or multiple devices, possibly in an array on a common substrate, with separate or common fluidic and electrical connections as may be necessary or advantageous.

Operation of the invention will now be described by way of overall reference to the figures and specific reference to Figure 5 which shows a diagrammatical view of a system incorporating the invention.

Substrate 10 is shown in which the wells 15 are illustrated diagrammatically. Address bus 17 provides a means of interconnecting the data lines from electrodes 16 to a micro processor 50. When a cell is present in well 15, as detected by electrodes 16, a signal indicating this is sent via address bus 17 to microprocessor 50. A separate function performed by the microprocessor increments a counter each time a cell is detected in well 15. Using predetermined look up table and/or an algorithm, microprocessor sends a signal along line L1 to pump 52. The pump may be replaced by a suitable

pressurising means or any suitable mechanism for displacing fluid such as electrostrictive means or electro-osmosis. This signal varies the pressure applied by pump 52 to the fluid present in the apparatus. The reason the fluid pressure is varied is so that as more cells enter wells 15 less pressure is required in order to drive cells into remaining vacant wells. If the pressure was maintained, due to the increase in impedance as wells become occupied, the pressure on one side of the substrate would rise and this would tend to urge cells through the wells. Alternatively or in addition to a very fine mesh (not shown) or other restrictive means may be applied to the backface of the wells which restrictive means permits the flow of fluid there through but prevent passage of cells. At the end of a cycle the restrictive means may be moved or removed thus permitting the cells to pass through the wells and a fresh charge of cells to be introduced. Cells are stored in a reservoir 54 and are introduced via valve 56 which is under control of the microprocessor 50.

The method will now be described with general reference to all the Figures. A cell 18 passes into well 15 and its presence is detected by proximity detectors. A signal is sent to a counter in microprocessor 50. The counter increments and the value stored therein may be used to modify one or more system parameters, for example pumping pressure. Electroporation then occurs either on the individual cell or simultaneously with one or more other cells.

The invention has been described by way of examples only. It will be understood variation may be made to the examples without departing from the scope of the invention.

CLAIMS

1. Apparatus for introducing a substance into an object comprising: means for introducing the substance into the object; and means for causing permeability of the wall of the object so as to permit said substance to enter said object; characterised in that the means for causing a permeability includes at least one electrode, dimensioned and arranged to permeate in the wall of the object upon application of a voltage pulse.

2. Apparatus according to claim 1 wherein a channel is provided through which an object passes or flows to a position where it is permeated, the channel is of the order of 50 μm, preferably the channel is less than 30 μm.

3. Apparatus according to claim 1 or 2 having a pump for urging objects into a location at which the voltage pulse is applied.

4. Apparatus according to any preceding claim wherein means is provided to restrain or locate an object in a desired position.

5. Apparatus according to any preceding claim having a proximity detector arranged to trigger the voltage pulse when the object is correctly located.

6. Apparatus according to any preceding claim formed on a semiconductor substrate.

7. Apparatus according to claim 6 wherein the semiconductor substrate includes silicon.

8. Apparatus according to any preceding claim wherein the means for introducing the substance into the object includes a syringe.

9. Apparatus according to any preceding claim including means for determining the state or condition of the object following transfection.

10. Apparatus according to claim 9, when dependent upon claim 7, wherein the means for determining the state or condition of the object includes an optical sensor and an infra-red source.

11. Apparatus according to any preceding claim including sorting means arranged to direct transfected objects to a first repository and non-transfected objects to a second repository.

12. A plurality of apparatuses, according to claim 1 arranged in an array.

13. Apparatus according to claim 12 wherein, in use, objects are supported in a fluid and means is provided to pressurise the fluid so as to urge the objects into locations whereat the walls of the objects may be made permeable.

14. A method of introducing a substance into an object comprising the steps of causing the wall of the object to become permeable so as to permit the substance to enter said object, and urging the substance and object towards one another so that the substance enters the object, characterised in that the object is made permeable by subjecting it to a voltage pulse.

Fig.1.

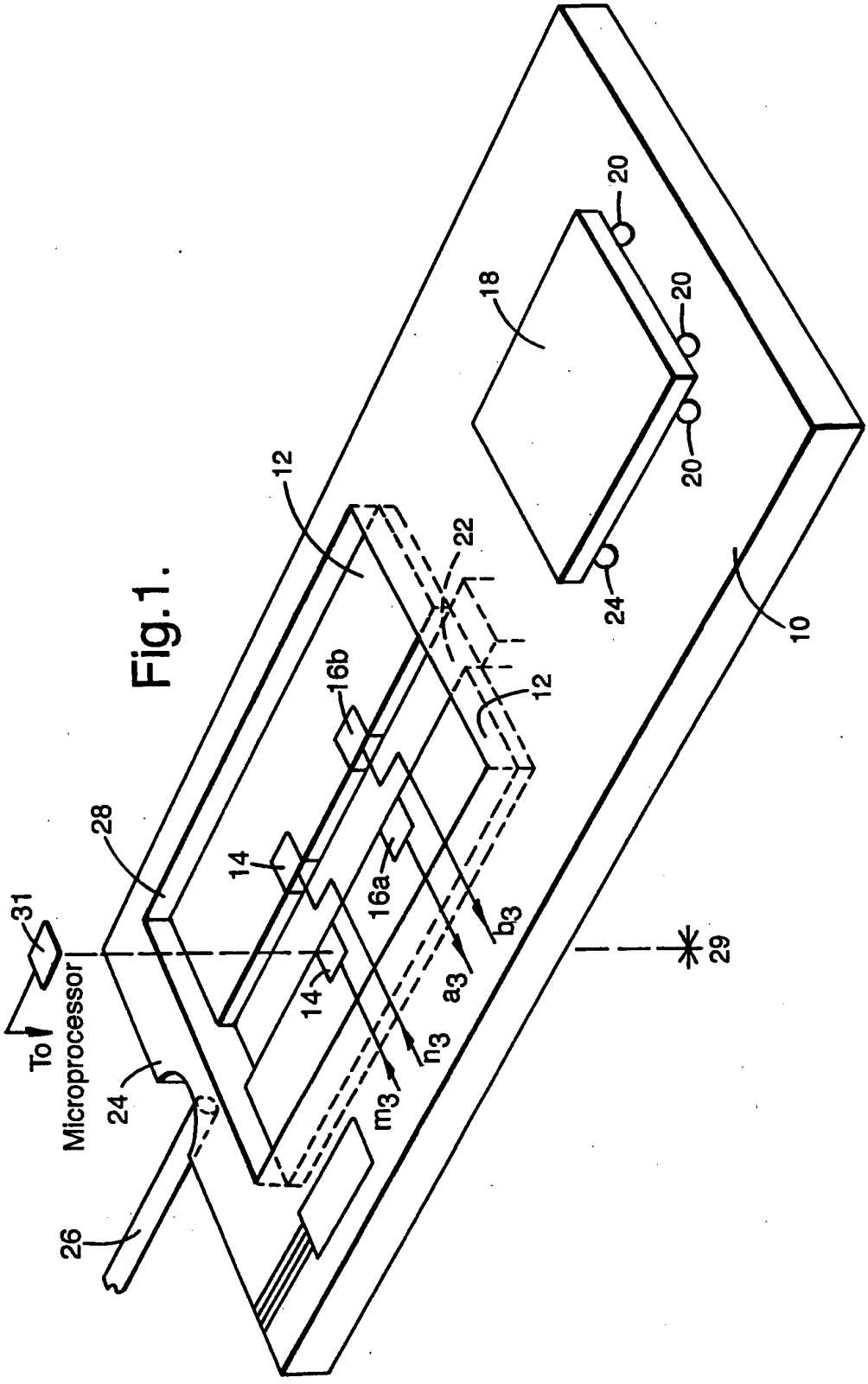
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Fig.2.

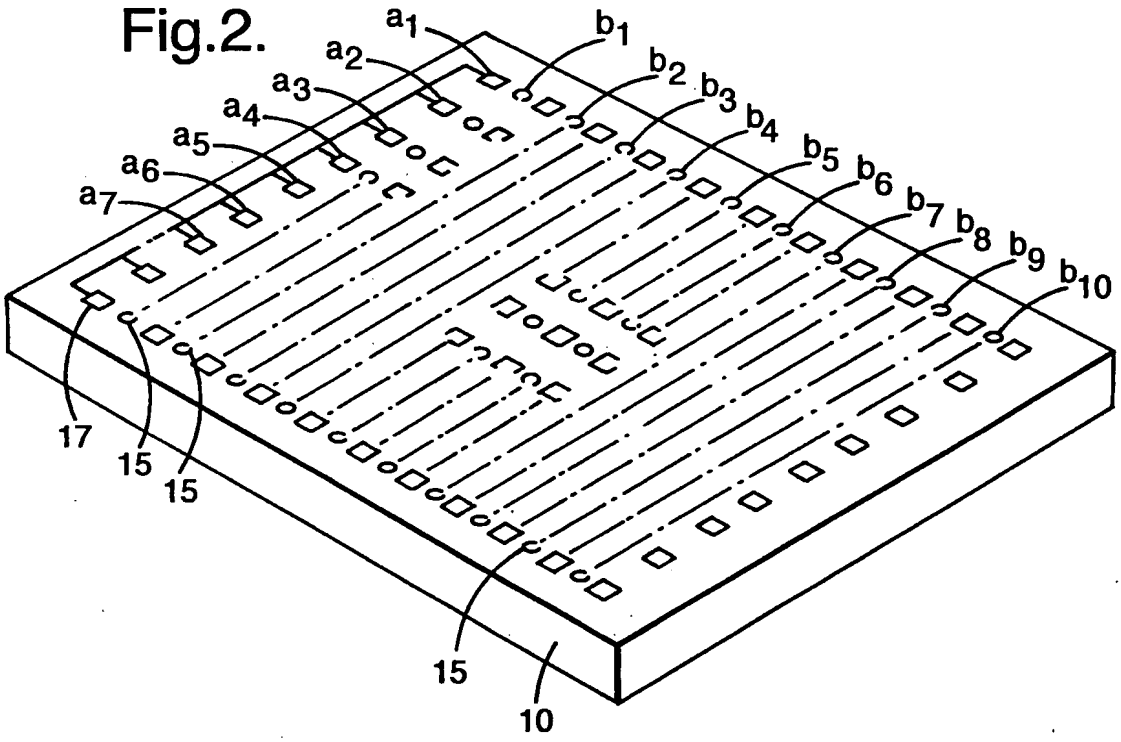
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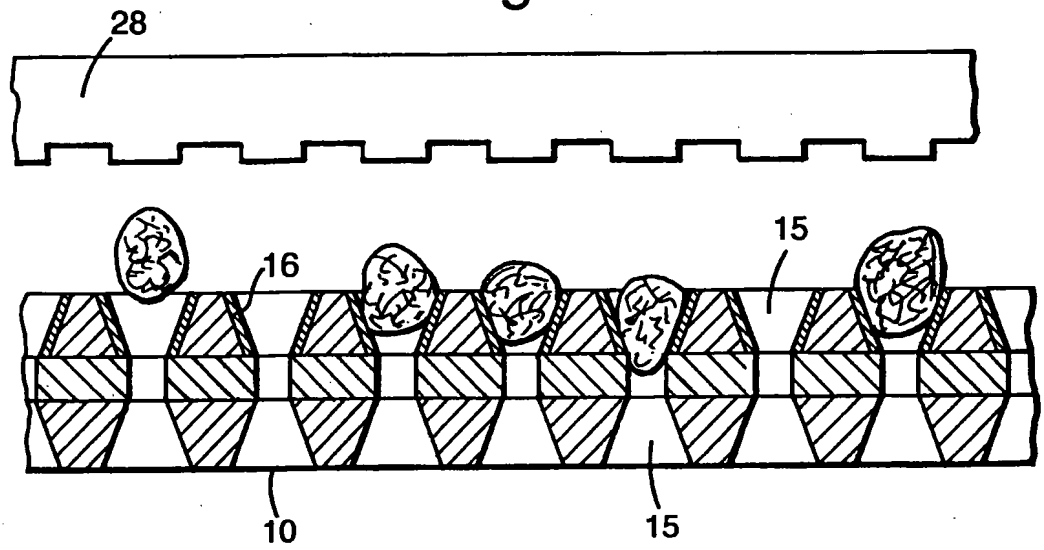
Fig.3.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15

Fig.4a.

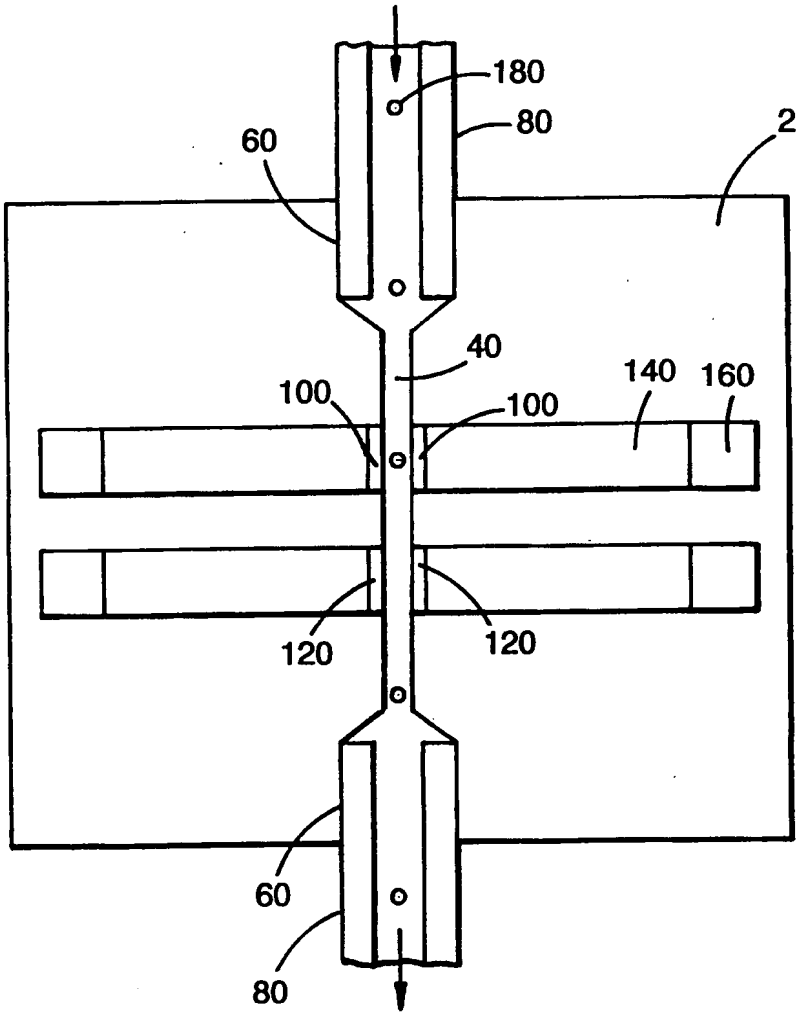


Fig.4b.

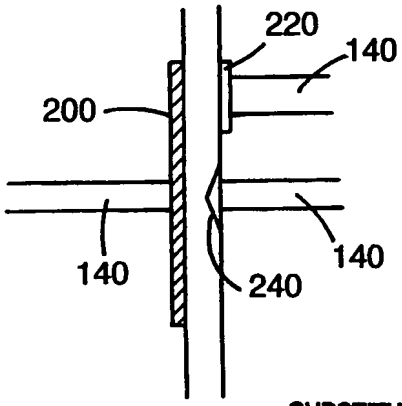


Fig.4c.

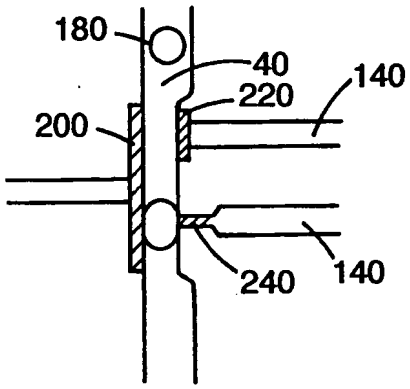


Fig.4d.

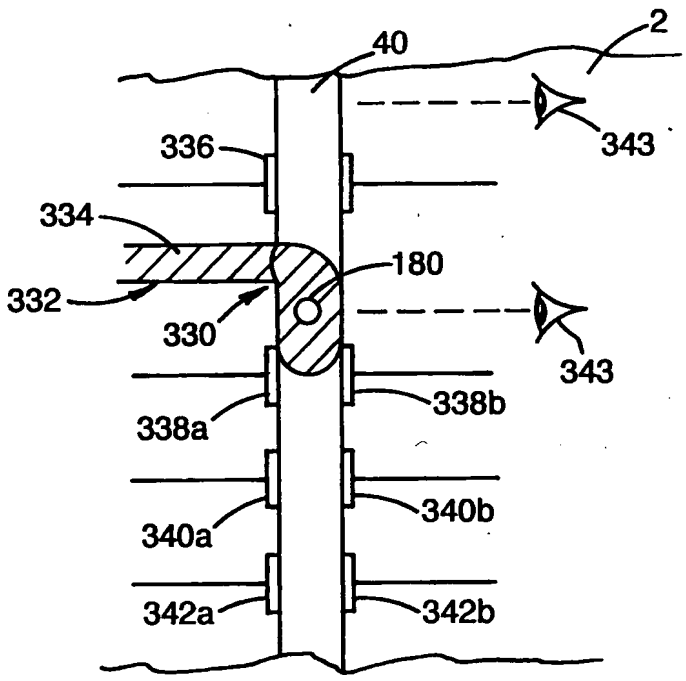


Fig.4e.

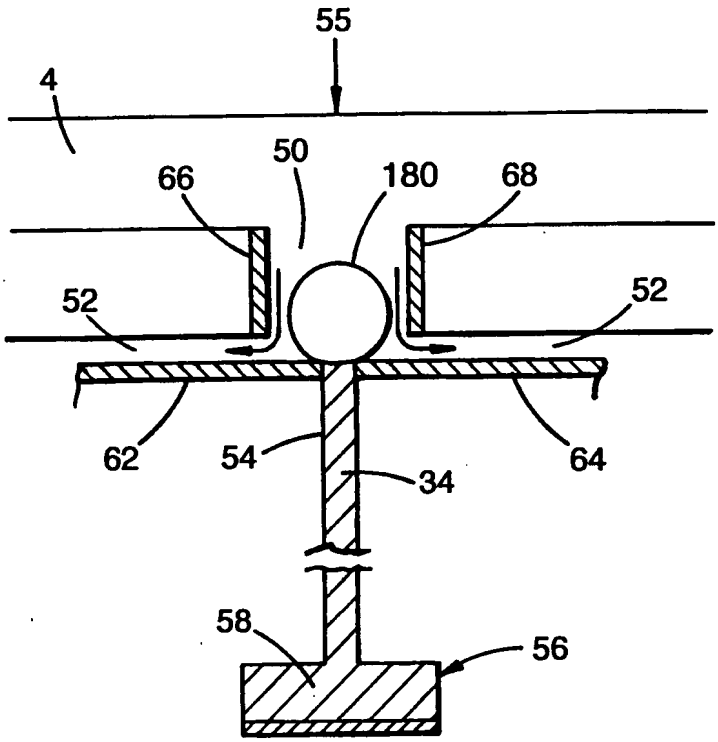


Fig.5.

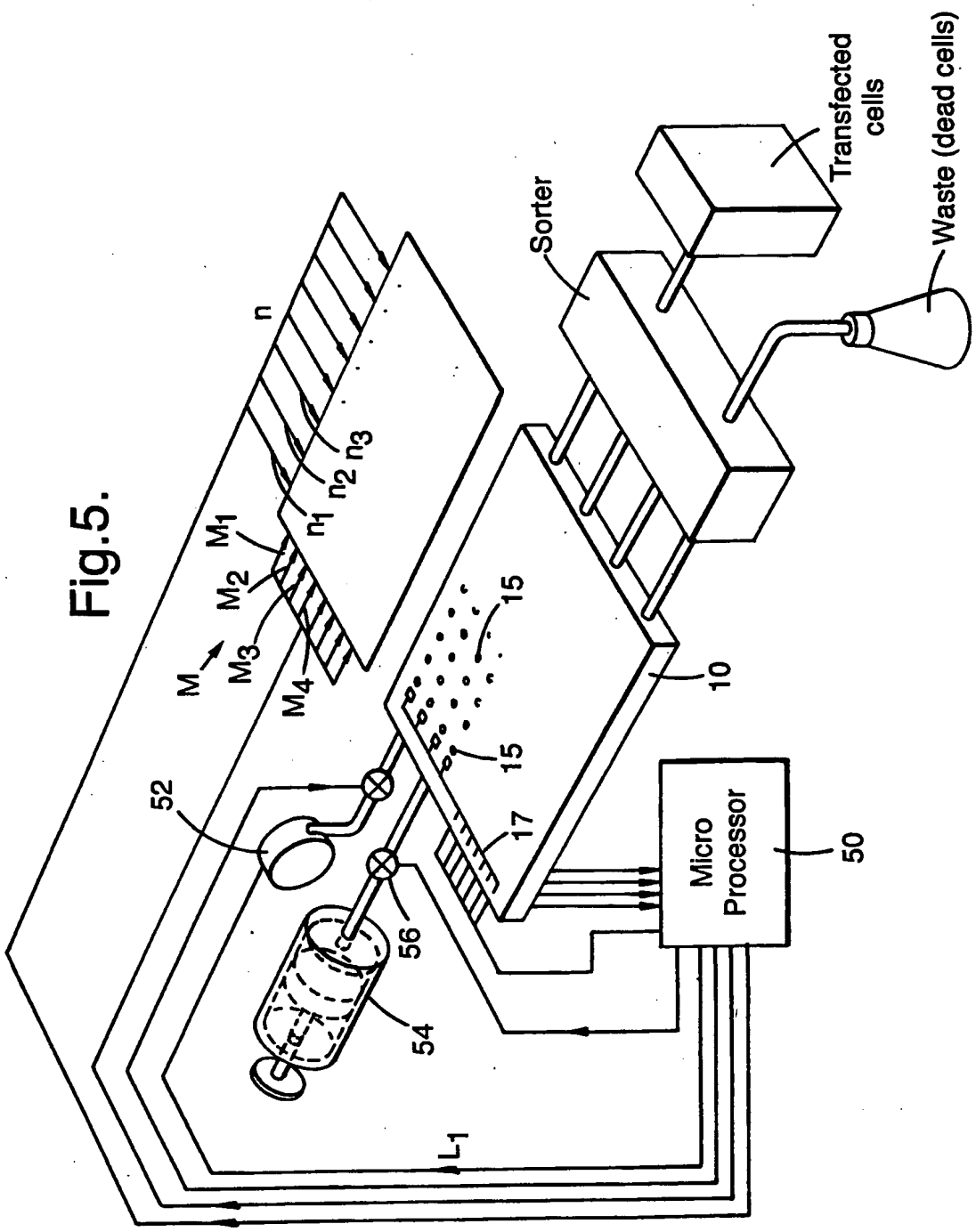
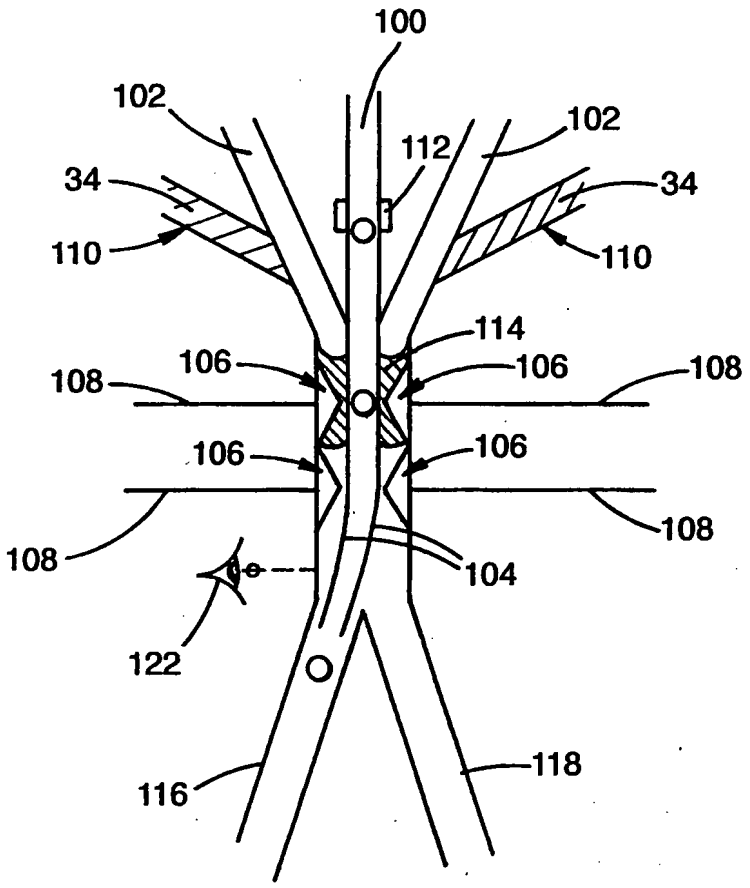

n
n1
n2
n3
M1
M2
M3
M4
M
52
56
54
L1
15
15
17
10
Micro Processor
50
Sorter
Transfected cells
Waste (dead cells)

Fig.6.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